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TITLE: GLOBAL EPIGENETIC CHANGES MAY UNDERLIE ETHNIC DIFFERENCES AND SUSCEPTIBILTY TO PROSTATE CANCER

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14. ABSTRACT **PURPOSE:** The purpose of the present study is to investigate genome-wide DNA methylation changes in prostate specimens from African-American (AA) and European-American (EA) men in order to elucidate the differential DNA methylation changes associated with prostate cancer disparity and identify novel biomarkers for early disease detection. **Results:** We examined the methylation status of a total of 7 normal prostate tissues and 3 prostate cancer tissue samples from AA men and compared it with 8 normal prostate tissues and 3 prostate cancer tissue samples from EA men using the Infunium 450K (484,968) CpG sites that corresponds to 21,221 genes in microarray (illumina). Pathway analysis of microarray data for the genes with altered methylation patterns showed the involvement of cancer related genes in networks of axonal guidance, RhoA signaling and androgen signaling in AA specimens versus genes involved in epithelial-mesenchymal transition in EA specimens. **Conclusion:** Our genome-wide methylation analysis suggests differential methylation of several genes in important signaling pathways associated with prostate cancer progression. On-going studies is to validate the illumina microarray results using the methyl-binding domain of MBD2 (qMBD-seq) technique in order to identify regions that are consistently differentially methylated in AA versus EA specimens. For several genes where antibodies are available we will validate expression in tissue microarray analysis using prostate tissues from AA and EA men who have undergone radical prostatectomy.

15. SUBJECT TERMS
GENOME-WIDE DNA METHYLATION CHANGES, PYROSEQUENCING, RT-PCR, WESTERN BLOT ANALYSIS, PROSTATE CANCER, AFRICAN AMERICAN , EUROPEAN-AMERICAN MEN

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INTRODUCTION

Prostate cancer (PCa) is a common malignancy and a leading cause of cancer death among men in the United States. Racial differences in PCa incidence and mortality are well documented. The incidence and mortality for PCa is about twofold higher in African-American (AA) in comparison with European-American (EA) men, with AA men experiencing among the highest rates worldwide[1]. African-American men have a 60% increased risk of developing prostate cancer, twice the risk of developing distant disease and twice the mortality relative to their European-American counterpart [2]. The disparity in PCa is believed to be a complex combination of socioeconomic factors, environment and genetics [3]. Genetic differences including mutation, loss or the amplification of different chromosomal regions may also play a major role in the disparity observed in prostate cancer incidence and mortality between AA and EA men. Recent advances in genomic studies have identified several genes involved in pathways relevant to prostate cancer biology; in particular, a number of genes with alleles which differ in frequency between AA and EA have been proposed as a genetic cause or contributor to the increased risk of PCa in AA. Genes that are involved in the sex steroid hormones and components of their signaling and metabolic pathways has been extensively studied in relation to prostate cancer susceptibility and shown to differ in AA and EA populations [4]. To date, single-nucleotide polymorphisms (SNPs) identified on 8q24 is well documented as risk alleles for AA men with prostate cancer [5]. However, the risk

variants are located in non-protein coding regions and the biological mechanisms underlying this association remain unclear.

In addition to the genomic defects associated with increased risk of prostate cancer, epigenetic defects such as DNA methylation changes and histone modifications are increasingly found to be associated with prostate disease prevalence. There is growing evidence to suggest that epigenetic DNA methylation changes affects gene expression in an age-dependent and tissue specific manner [6]. We have recently demonstrated that indeed CpG hypermethylation increases with age in the normal human prostate tissues [7]. Such age-dependent DNA methylation changes can alter cell physiology and possibly, predispose prostate cells to neoplastic transformation. Also in many cases, aberrant methylation precedes full-blown malignancy and can often be found in non-cancerous tissues; in the prostate, hypermethylation of the GSTP1 CpG has been detected in PIA lesions [8]. DNA methylation occurs at CpG sites in the human genome and involves the addition of a methyl group to the 5'-carbon of cytosine, forming 5-methylcytosine (5-meC; [9, 10]). Since the recognition that the GSTP1 CpG island was frequently hypermethylated in PCa, more than 40 genes have been reported to be targets of DNA hypermethylation-associated epigenetic gene silencing in PCa cells [11]. Ethnic group-related differences in CpG hypermethylation has also been identified for several genes. One study demonstrated that GSTP1 hypermethylation was significantly higher in PCa samples from AA men in comparison with EA and Asians [12]. Another study found higher frequency of hypermethylation of cell adhesion molecule

(CD44) hypermethylation in prostate cancer tissues from AA in comparison to EA [13] suggesting that inactivation by CpG methylation may play a role in the disparity associated with prostate carcinogenesis. Our preliminary data demonstrate higher methylation of several genes in prostate tissue samples from AA in comparison with their EA counterparts [14]. The current literature suggests a complex mechanism of epigenetic regulation in prostate cancer including DNA methylation changes that can lead to either gene silencing or the activation of key regulatory genes in the disease pathway. Underlying this aberrant DNA methylation is the accumulating body of data hinting that normal prostate cells may be subjected to a relentless barrage of genome-damaging stresses due to both exogenous and endogenous carcinogens, with damage accumulating over time and age. Thus aberrant epigenetic DNA methylation changes may represent the integration of environmental or lifestyle exposures and genetic predisposition to prostate cancer. Such events may differ between individuals belonging the same ethnic group or individuals belonging to different ethnic groups. Thus the elucidation of genome-wide DNA methylation changes in prostate tissues from different ethnic groups would contribute to our understanding of the molecular mechanisms underlying prostate cancer disparity and potentially lead to the identification of “ethnic sensitive” biomarkers for early disease detection. We know that there are different thresholds for AA versus EA men for PSA screening. Therefore other markers such as DNA methylated genes that can clarify such ethnic sensitive screening strategies would also be helpful.

Furthermore, differential methylation changes could also lead to identification of potential novel therapeutic targets for prostate cancer treatment.

In this study we sought to investigate whether differential DNA methylation changes may represent an integration of lifestyle and genetic predisposing factors to create a more aggressive disease milieu in African American patients.

BODY

As outlined in our Statement of work, we seek to accomplish 3 main tasks during the 3 years of funding. Specifically for year 2 (months 12-24), the aims that we sought to accomplish are the following: Aim 1: **Evaluation of genome-wide differences in DNA methylation in prostate tissues from African American (AA) versus Caucasian (EA) Men (months 0-24).** This will be accomplished by 2 tasks**: Task 1**: Identify, process and isolate DNA from fresh frozen prostate tissues from AA and EA subjects as described in proposal and **Task 2**: Carry out genome-wide DNA methylation analysis on samples. Aim 2: **Validation and quantification of genomic DNA methylation changes in prostate tissue samples from AA versus EA men (month 0- 30).** This will be accomplished by 5 tasks: **Task 1**: Acquisition of prostate tissues from AA and EA men. Obtain prostate tissues from both AA and EA men who have undergone radical prostatectomy (200 formalin fixed paraffin embedded (FFPE) samples from each racial group matched for age, PSA and Gleason Score) (month 6-24). **Task 2**: Extraction of DNA from specimens obtained in task 1 (moth 8-30). **Task**

3: Screening and validation of 20 differentially methylated genes and 5 that have been previously identified through preliminary studies (moth 12-30). **Task 4**: Design bisulfite primers based on bisulfite-converted sequence from the CpG island. For each gene, optimize pyrosequencing reaction by designing 2 or 3 pyrosequencing assays (month 14-16) and **Task 5**: Perform large scale and high throughput pyrosequencing reaction for each methylated gene using modified DNA from prostate tissues and analyze data (month 17-30).

We have made substantial progress at the end of this second year of funding period as detailed below. A manuscript describing the "Identification of novel DNA-methylated genes that correlate with human prostate cancer and high-grade prostatic intraepithelial neoplasia" was recently published in Prostate Cancer and Prostatic Diseases (Jul 30; 2013/Epub ahead of print; PMID: 23896626), a copy of the article is attached below. In addition two abstracts from this work were presented at the American Association for Cancer Research 2013, Annual Meeting in Washington D.C. (a copy of each abstract is attached below).

Specific Aim 1: Evaluation of genome-wide differences in DNA methylation in prostate tissues from African- American versus European-American Men (months 12 – 24):

Task 1: Identify, process and isolate DNA from fresh frozen prostate tissues from AA and EA subjects as described in proposal:

We have now completed these activities, yielding high quality genomic DNA, and genome-wide analyses of these materials are well underway.

Task 2: Carry out genome-wide DNA methylation analysis on samples.

After creating mature computational and bioinformatics tools, and also optimizing the qMBD-seq approach in year one of the progress, we have now nearly completed data collection of the DNA from AA and EA subjects for whom DNA was isolated in Task 1. We are in the final stages of data collection and analysis, and are still blinded to the race information so that we can make unbiased methylation measurements. The data are of high quality, with each sample achieving a minimum of 10,000,000 aligned tags in the enriched-methylated and total-input fraction. These data have now been carried through alignment, peak calling to identify methylated regions, and are now being processed through our computational approaches for quantitative analysis. Once these activities are completed in the next one to two months, we will “unmask” race information and carry out comparisons between groups. See **Figure 1** below to see a representation of the data on a handful of representative tumor-normal pairs.

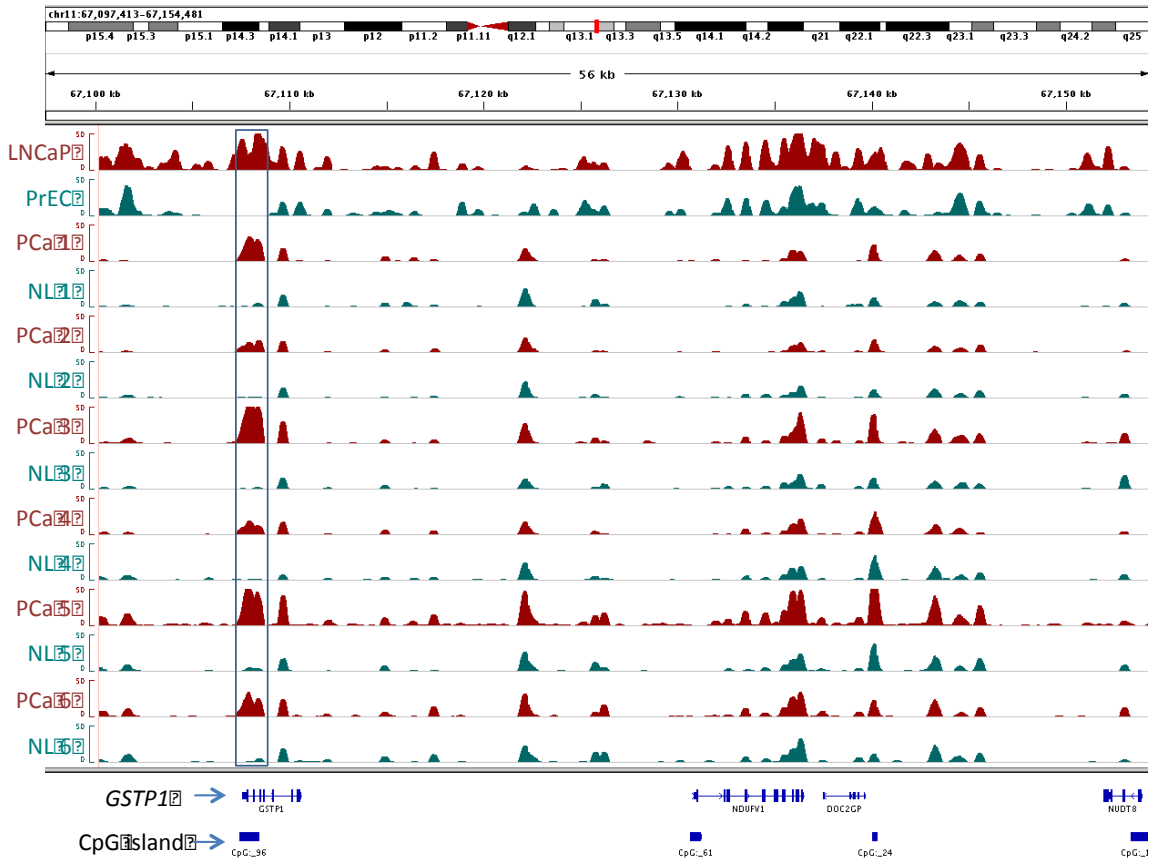


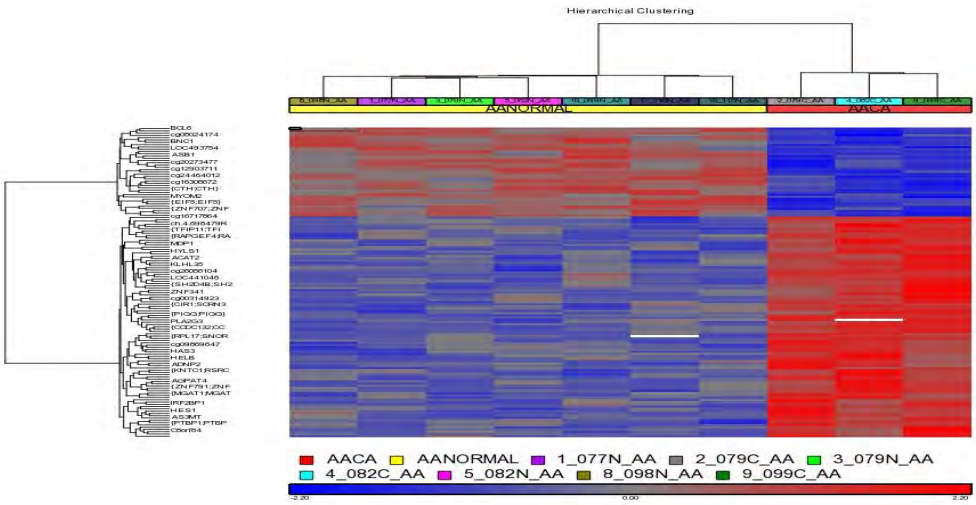
Figure 1. Representative results from qMBD-seq analyses. Shown is a region surrounding the GSTP1 gene. This region was selected because it has now been well-appreciated as a highly frequently hypermethylated gene in prostate cancer in nearly all cases (>90% sensitivity and ~100% specificity). Red tracks are tumors, and green tracks are matched normal specimens, with the top two tracks from the LNCaP prostate cancer cell line and PrEC normal prostate epithelial cells in primary culture. Note that all tumors show hypermethylation of the boxed regions in the tumors and not normals. The boxed region shows the CpG island of the human GSTP1 gene, which is consistently hypermethylated in all tumor samples, but in none of the normal samples. This representative region also shows the exquisitely high resolution of the approach since it can differentiate between the cancer-specific hypermethylated region and the regions that are normally methylated that are in the downstream exons and intergenic regions.

In a parallel approach, we have examined the genome-wide methylation pattern in prostate tissue samples derived from African-American men in comparison to European-American men using the Infunium DNA methylation array (484,968 CpG sites that corresponds to 21,221 genes; illumina) analysis. The 450K microarray includes CpG islands/shores/shelves/open sea, non-coding RNA and

sites surrounding the transcription start sites for coding genes, but also for the corresponding gene bodies and the 3'-UTR.

For the genome-wide large-scale DNA methylation analysis, we used genomic DNA derived from a total of 7 normal prostate tissues and 3 prostate cancer tissue samples from AA men and compared it with 8 normal prostate tissues and 3 prostate cancer tissue samples from EA men. Unsupervised hierarchical clustering analysis of all samples (**Figure 2**) show heterogeneity in individual prostate tissue samples. However, for each ethnic group, normal prostate tissue samples clustered together, whereas most prostate cancer cases generally clustered together.

A.



B.

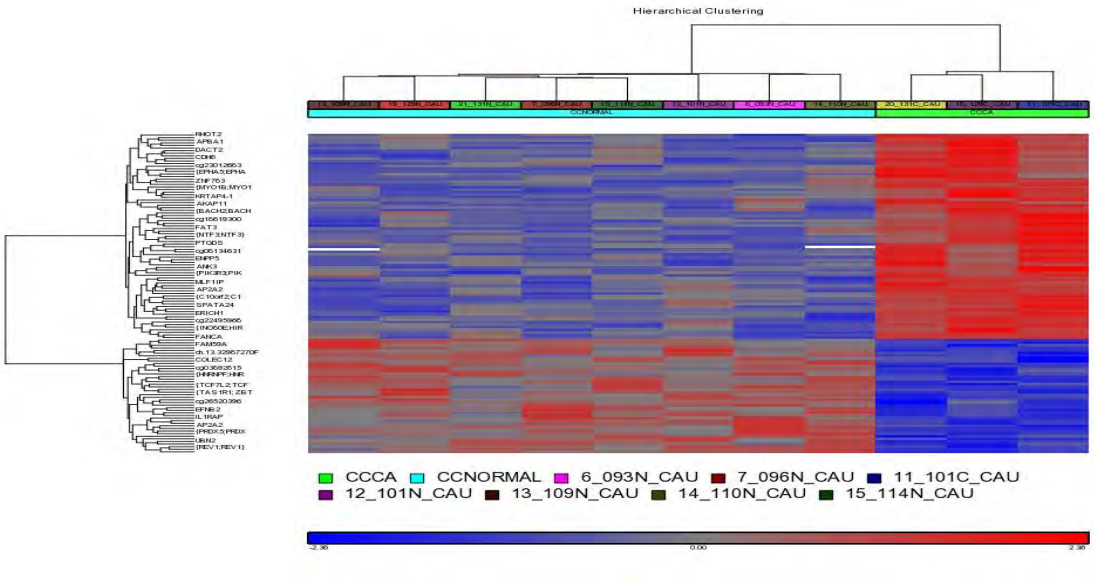


Figure 2. Hierarchical cluster analysis of methylation findings from the microarray data. DNA methylation hierarchical clustering shows high within-sample group similarity and between-samples heterogeneity. These analyses were done using Partek genome studio software with 450 gene probes showing greatest variability across all samples. CpG methylation differences were considered significant above a cut-off p value < 10^{-3} and 0.2-fold change in the β (beta) value. The scale shows β value for methylation score. **A.** Prostate tissue samples from African-American men. **B.** Prostate tissue samples from European-American men.

Pathway analysis of the genes with altered methylation patterns identified top canonical pathways for the involvement of cancer related network for genes involved in axonal guidance, antigen presentation, androgen signaling and protein ubiquitination pathways in prostate cancer tissues compared with normal prostate tissues obtained from African-American men. On the other hand, the top canonical pathways identified in prostate cancer tissues compared with normal prostate tissues obtained from European-American men are genes involved in epithelial-mesenchymal transition, p53 signaling, nucleotide sugars metabolism and germ cell-sertoli cell junction signaling pathways.

Aim 2: Validation and quantification of genomic DNA methylation changes in prostate tissue samples from African American versus European-American men (months 0 – 30)

All the 5 tasks involved in this aim are on-going. Several novel genes have been identified by our genome-wide methylation analysis and validated in prostate cell lines and tissue samples from both African-American and European-American published as shown below (**Figure 3 and 4**) and also published in the Prostate Cancer and Prostatic Diseases (Jul 30, 2013 [Epub ahead of print]/PMID: 23896626; see attachment). In addition, other novel genes are currently been validated.

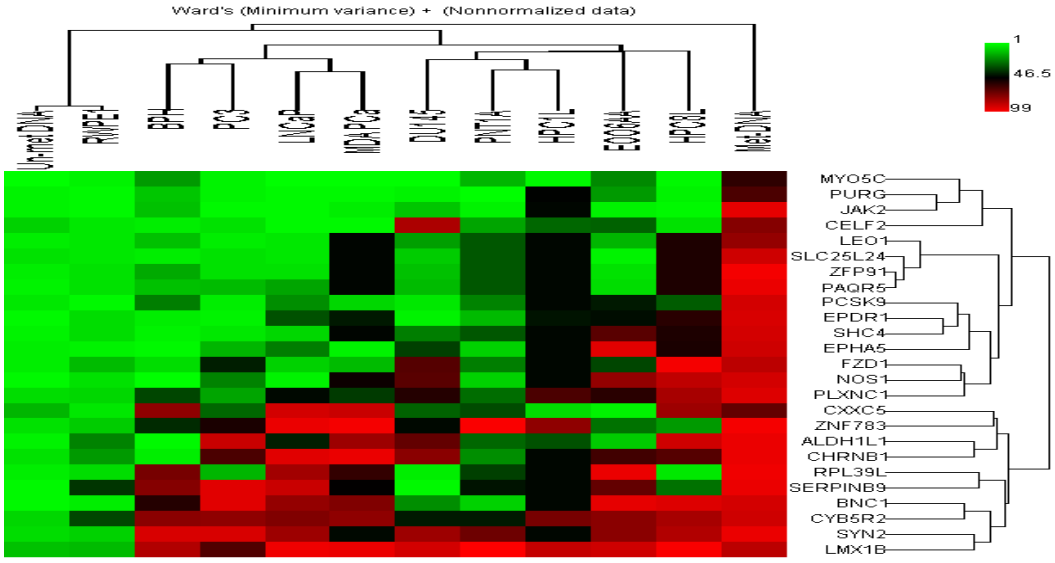


Figure 3. Hierarchical cluster analysis of gene validation of 25 novel selected promoter associated DNA methylated genes in a panel of prostate cell lines: RWPE1: Primary immortalized prostatic epithelial cells; PNT1A, primary immortalized epithelial cells; BPH; EA prostate cancer cell lines are LNCaP, PC3 and DU145; AA prostate cancer cell lines are MDA PCa2b, HPC1L, HPC8L and E006AA.

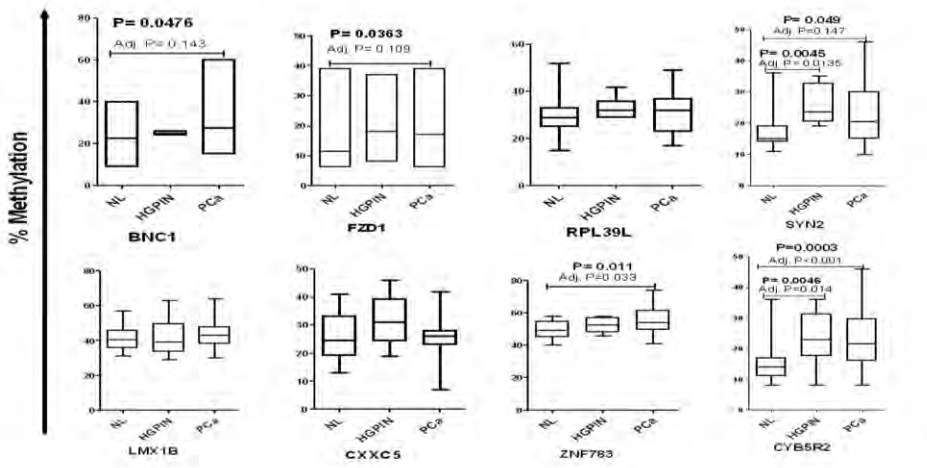


Figure 4- Quantitative DNA methylation analysis in human prostate tissues is shown in the box plot. The percent DNA methylation levels of promoter CpG islands were analyzed in bisulfite-modified genomic DNA extracted from matched pairs of normal (NL), HGPIN and prostate cancer (CA) tissue samples obtained from EA cancer patients who had undergone radical prostatectomy (Age 45-67 years old). *Y* axis, percentage of methylated cytosines in the samples as obtained from pyrosequencing. *X* axis, normal, HGPIN and CA tissues. The box plot describes the median, inter-quartile range and maximum/minimum methylation. P-value where significant ($P < 0.05$; Mann-Whitney *t* test) is shown.

Aim 3: Comprehensive evaluation of the biological function of novel genes that are differentially methylated in prostate tissue samples in African-American versus European-American men.

We have identified several candidate genes including Basonuclin 1 (*BNC*1) as novel genes that are frequently methylated and whose methylation are closely related to inactivation of gene expression in prostate cancer cell lines. Basonuclin1 is a zinc finger transcription factor that interacts with a subset of promoters of genes transcribed by both RNA polymerase-I and -II and has roles in maintaining ribosomal biogenesis and the proliferative potential of immature epithelial cells. We found that the expression of BNC1 negatively correlated with

the degree of methylation at the CpG island in prostate tumor samples compared to benign prostate tissues. The forced-expression of BNC1 significantly decreased prostate cancer cell proliferation whereas the siRNA mediated BNC1 knock-down increased prostate cancer cell proliferation. Overall, our data suggest a potential tumor suppressor role for BNC1 that includes regulation of cell proliferation and cell-cell interaction in prostate carcinogenesis.

Methodology

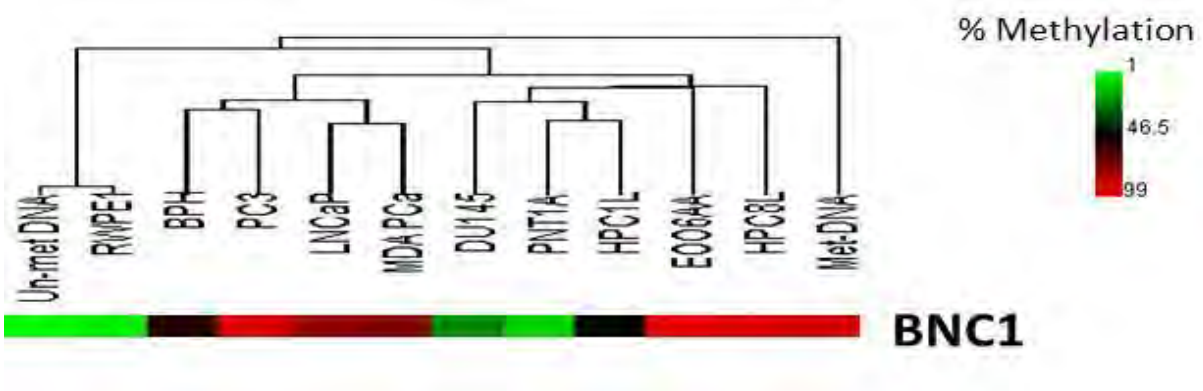
***Tissues*:** Normal and prostate cancer tissues (N=7) were obtained with informed consent from Baylor College SPORE tissue bank. The pathological status was confirmed before H & E staining, and the cancer samples had a tumor cell percentage of 70%-100% with Gleason scores of 6-8. Immunohistochemistry analysis was carried out by Pantomics Inc. using *BNC*1 antibodies from Santa Cruz.

Pyrosequencing: High molecular weight genomic DNA extracted from prostate tissues were modified by sodium bisulfite treatment. The bisulfite treated DNA were used in a PCR reaction with bisulfite PCR primers. The reverse primer was biotinylated. The PCR product was immobilized on streptavidin-sepharose beads, washed, denatured and the biotinylated strands released into an annealing buffer containing sequencing primer. Pyrosequencing was performed using PSQ 96HS machine.

***Phenotypic characterization*:** Gain-or-loss functional studies was carried out by transiently transfecting BNC1 plasmid or siRNA vectors (Origene) into prostate cancer cells by lipofection. Cell proliferation was analyzed by coulter counter and gene expression by quantitative RT-PCR using Taqman probes. Cell apoptosis was detected using the BD Pharmingen Annexin V FITC apoptosis detection kit and a Cellometer.

Results:

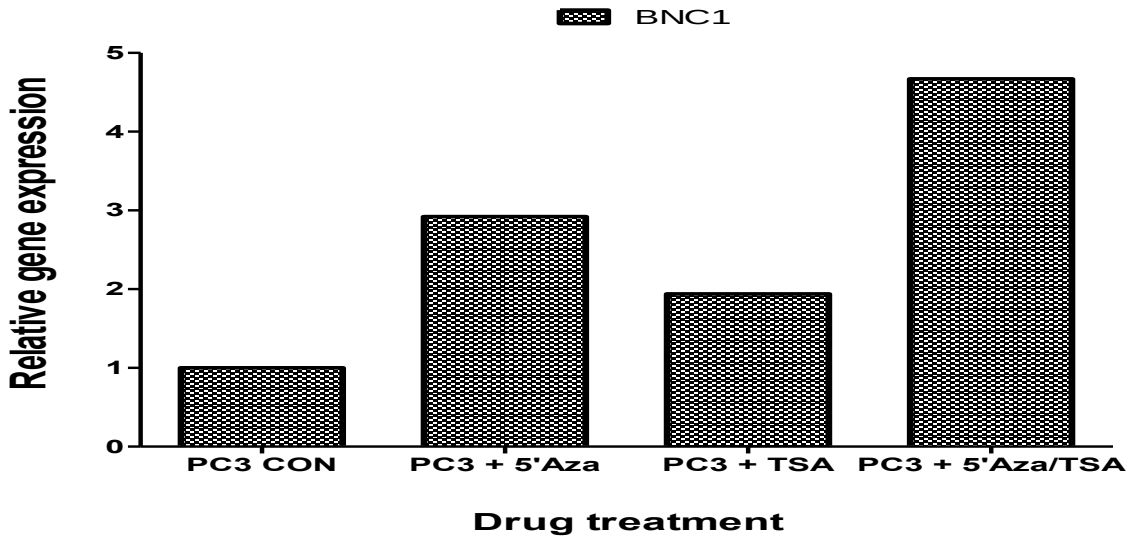
A. BNC1 Methylation analysis in prostate cell lines



We observed differential methylation level for the *BNC*1 promoter CpG Island in prostate cell lines; The normal prostatic primary epithelial cell lines (RWPE1 and PNT1A) derived from European-American men showed low percent methylation whereas the prostate cancer cell lines derived from African-American men (HPC1L, E006AA, MDA-PCa) showed high percentage of methylation. The prostate cancer cell lines derived from European-American men (PC3, LNCaP and DU145) showed moderate to high percentage of methylation whereas the

BPH (Benign Prostatic Hyperplasia) cell line demonstrated moderate percentage of methylation.

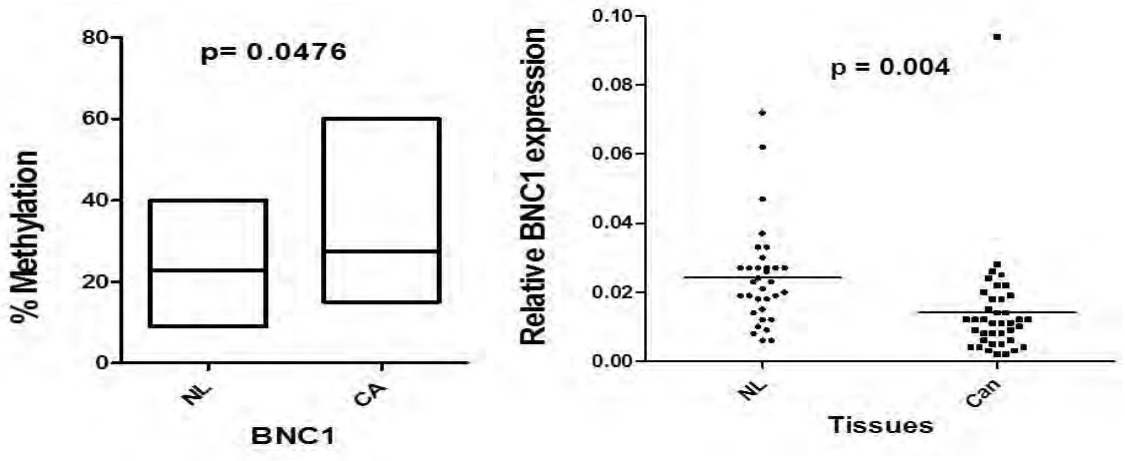
B. Demethylation and gene expression in response to 5'-aza-2 deoxcytidine (5 uM), Trichostatin A (250nM) or both.



We next treated the PC3 prostate cancer cell lines with the demethylating agent 5'-Aza-dC with or without the histone deacetylase inhibitor, trachostatin A (TSA) and carried out semi-quantitative RT-PCR analysis using untreated (PC3 con) and treated cells to assess whether promoter CpG island hypermethylation of the *BNC*1 gene was closely associated with gene expression in the newly identified cancer specific methylated genes. Treatment with 5'-Aza-dC caused about 3-fold increase in the mRNA transcript levels for BNC1. Treatment with TSA also increased the mRNA transcript levels for BNC1 and the combined 5'Aza-dC and TSA treatment significantly increased the mRNA transcripts for all genes tested in PC3 cells. The results indicate that epigenetic mechanisms including DNA

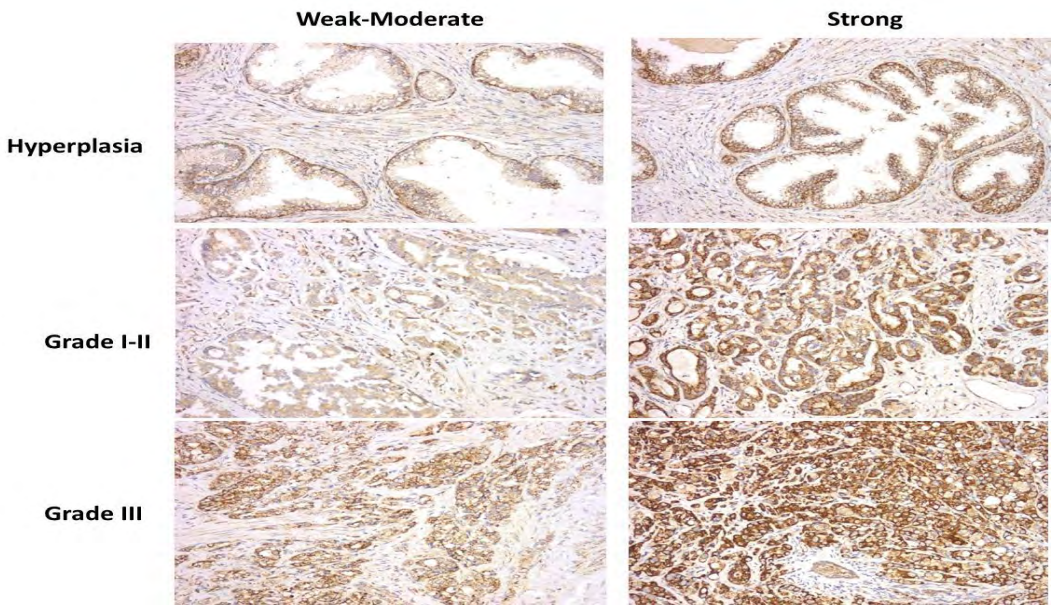
methylation are likely involved in the reduced expression of BNC1 in prostate cancer cells.

C. **BNC1 Methylation and expression in human prostate tissues**



To investigate if methylation is associated with the silencing of *BNC*1 gene in prostate cancer tissues, we performed expression analysis by quantitative RT-PCR. The results showed a significantly higher level of gene expression (*P* = 0.0006) in the matched normal prostate tissues (NL) when compared with the prostate cancer (PCa) tissues. Our data suggest an inverse association between DNA methylation and gene expression for *BNC*1. We observed that in cancer samples that showed higher methylation, this was associated with low levels of gene expression, whereas the normal prostate samples had lower methylation and higher gene expression level to indicate that methylation leads to some loss of gene expression.

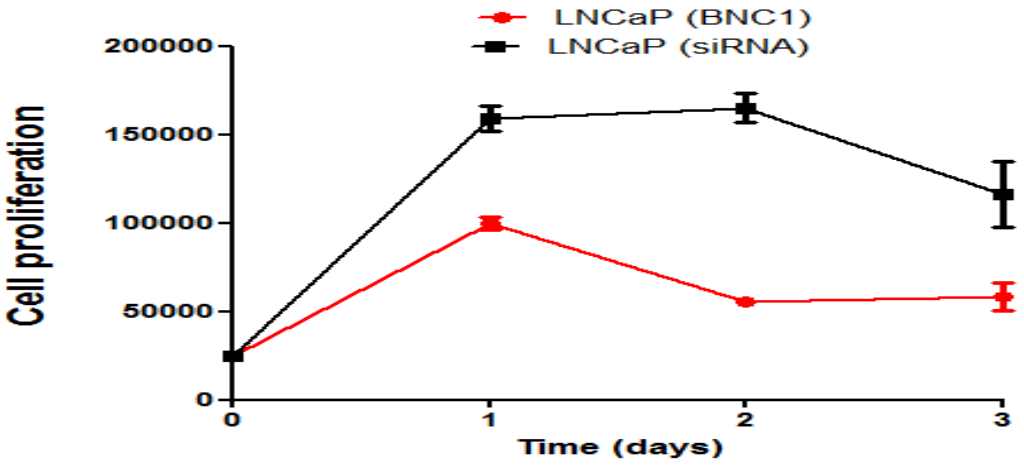
D. **IHC Staining of BNC1 antibodies in Prostate Cancer Tissue Microarray**

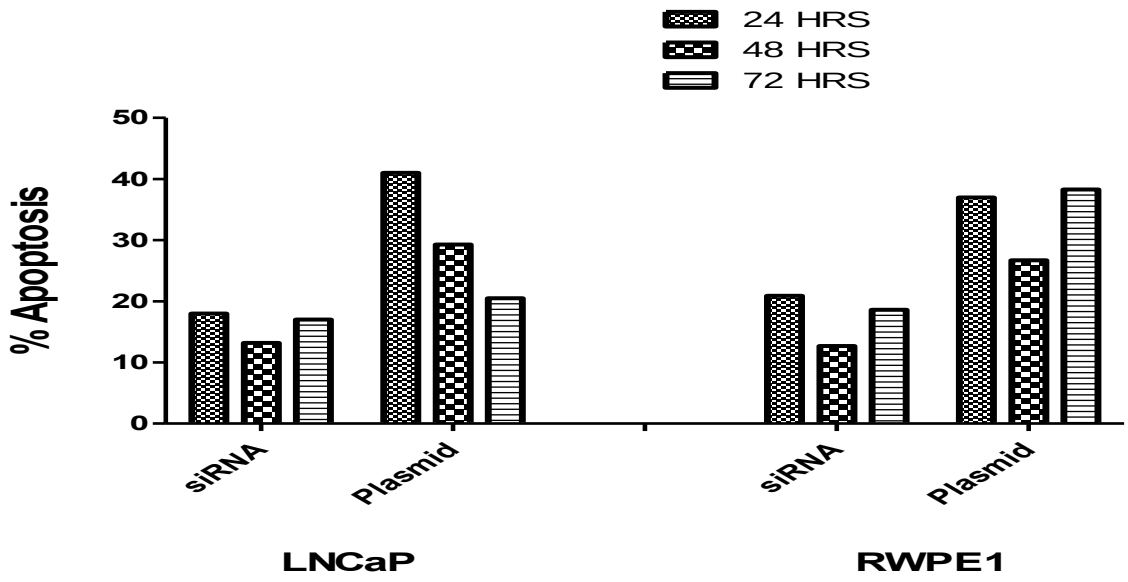


To determine whether BNC1 protein is decreased in prostate cancers, we analyzed a total of 48 prostate cancer cases with progressive changes by immunohistochemistry using goat polyclonal antibody to *BNC*1. The tissue microarrays (TMA) used was obtained from Pantomics, Inc. Briefly, all the cases in the TMA showed modetate to strong cytoplasmic staining to the BNC1 antibody. Weak to moderate cytoplasimc staining was also noted in stromal components. In hyperplasia cases, strong staining was mainly noted in the myoepithelial cells of the glandular structures. In the prostate cancer, the staining was evenly distributed among the tumor cells, and most of the cases showed strong staining. Four out of the 17 of the grade I to grade II showed very strong staining, while 7 out of 14 of the grade III showed very strong staining to the antibody. Overall, the staining intensity seemed to positively correlate with the

grade and Gleason scores of the tumors. However, it should be noted that a significant fraction of the prostate cancers showed moderate to weak staining. Therefore statistical analysis is needed to verify these results. The results suggest that although BNC1 decreased expression is seen in a fraction of prostate cancers, loss of BNC1 is clearly not required in all prostate cancers.

E. BNC1 induced cell proliferation and apoptosis





To ascertain the biological effect of BNC1 expression in human prostate cancer cells, pCMV6-BNC1 (encoding the full length of *BNC*1 sequence) or the SiRNA-BNC1 was transiently transfected into the human prostate cancer cell lines LNCaP. The forced expression of BNC1 inhibited LNCaP cell proliferation, whereas the knockdown of BNC1 expression by siRNA increased cell proliferation. Thus in prostate cancer cells, transiently increased BNC1 expression markedly inhibits proliferation.

We examined where BNC1 expression might mediate increase apoptosis in prostate cancer cells. Using transient transfections of prostate cancer cell lines with either the BNC1 expression plasmid or siRNA-BNC1, we determined whether BNC1 could affect apoptosis in prostate cancer cell lines. Using Annexin V/PI apoptotic assay, we observed about 2-fold increased apoptosis in prostate cancer cells transfected with the BNC1 plasmid when comparison with the siRNA transfection. This finding indicates that BNC1 could be a pro-apoptotic factor for

prostate cancer cell proliferation. Ongoing study is to investigate the signal transduction pathways mediated by BNC1 in prostate cancer.

KEY RESEARCH ACCOMPLISHMENTS:

- We have nearly completed all of the data collection and specimen level analysis of the genome-wide qMBD-seq experiments with AA and EA specimens. The data are of very high quality, with every specimen yielding high aligned tag-density for methylated regions. In the coming months, we will complete this process and carry out differential analysis between EA and AA specimens to identify regions that are consistently differentially methylated in EA specimens compare to AA specimens.
- We have used the illumina infunium 450K genome-wide methylation array to investigate the methylation profiles in prostate tissue samples from African-American men in comparison with European-American men. Hierarchical cluster analysis shows differences in the methylation patterns the African-American samples compared to the European-American samples with top canonical pathways canonical pathways identified the involvement of cancer related network for genes involved in axonal guidance, antigen presentation, androgen signaling and protein ubiquitination pathways in prostate cancer tissues compared with normal prostate tissues in African-American biospecimen. On the other hand, the top canonical pathways identified in prostate cancer tissues compared with normal prostate tissues in European-American biospecimen are

genes involved in epithelial-mesenchymal transition, p53 signaling, nucleotide sugars metabolism andgerm cell-sertoli cell junction signaling pathways.

- We have identified Basonuclin1 (*BNC*1) expression to be down-regulated in human prostate cancer tissues in comparison with benign prostate tissues by aberrant DNA methylation. The transient forced-expression of *BNC*1 caused growth inhibition and increased cell apoptosis suggesting a potential tumor suppressor function for BNC1 in prostate cancer. Cytoplasmic staining of BNC1 positively correlated with tumor grade and Gleason score, suggesting that BNC1 can be explored as a marker for prostate cancer disease progression.

REPORTABLE OUTCOMES:

- P01 sub-project entitled, “Curbing prostate cancer overtreatment through noninvasive epigenetic tests,” developed in part by using preliminary data supported by this project, has been submitted to NCI.
- Development of TMA with matched AA and EA prostate cancer specimens, through leveraging of funding from the DOD Prostate Cancer Biospecimen Network, through collaboration with Bruce Trock, and Angelo De Marzo.
- Manuscript entitled “Identification of novel DNA-methylated genes that correlate with human prostate cancer and high-grade prostatic

intraepithelial neoplasia" has been published in Prostate Cancer and Prostatic Diseases Jul 30, 2013 [Epub ahead of print/PMID: 23896626].

- ❖ AACR Annual Meeting: Abstract title "Functional characterization of Basonuclin 1 (BNC1): a novel tumor suppressor gene commonly downregulated in human prostate cancer" 2013. Washington, D.C.
- ❖ AACR Annual Meeting: Abstract title "ZNF783, a novel zinc finger protein has tumor suppressor function in prostate cancer" 2013. Washington, D.C.

CONCLUSION:

Our preliminary data using the Infunium 450K methylation array platform has demonstrated proof of principle that the quantification of methylation status by genome-wide arrays can be collaborated by single-gene based analysis such as pyrosequencing.

Our on-going genome-wide methylation technology is the qMBD-Seq approach that features use of the methyl-binding domain of the MBD2 protein (MBD2-MBD) to bind and enrich methylated DNA fragments with high avidity and specificity. The advantage of our qMBD-sequencing approach over other genome-wide approaches is that we can obtain genome-wide methylation data without bias to specific regions, with this in mind we are now ready to compare DNA methylation levels between African-American and European-American prostate cancer tissues in a genome-wide level.

FUTURE WORK WILL FOCUS ON:

- We will complete the analysis of the genome-wide qMBD-seq data to obtain regions that are differentially methylated between AA and EA prostate cancer specimens. Once this is completed, we will carry these forward for the validation studies using the matched AA and EA samples that we have now collected and made a TMA from. We will also characterize the most promising of these functionally in prostate cancer models.
- **Aim 3**- Comprehensive evaluation of the biological function of novel genes differentially methylated in prostate tissue samples from African American versus Caucasian men (months 24-36). We have identified several novel methylated genes including ABCC4, AMICA1, EPHA2, PLXNA1 and PRKCE that shows differential methylation frequency in prostate tissue samples from African-American men in comparison with their European-American counterparts. We will carry out gain-or-loss of functional studies using forced expression or gene knockdown (by siRNA/shRNA) of novel gene(s) with potential regulatory function in prostate cancer. To accomplish this, we will **o**btain the full length cDNA EST clone from Image consortium or by PCR amplification and clone it the cDNA into a suitable vector such as pCEP4 or pcDNA (Invitrogen) or purchase recombinant plasmid expressing gene of interest where commercially available. We will use the recombinant vector or siRNA/shRNA in transient and stable transfections where appropriate of pNT1A, DU145, PC3 and LNCaP. We will confirm over-expression or down-regulation at the mRNA level by quantitative

RT-PCR analysis and confirm protein expression of transfected cells by Western blot analysis by gene-specific antibody or to a vector-specific tag. Transfected cell number will then be determined by direct counting with a Coulter counter each day over a 6 day period. If novel gene is a negative regulator of prostate cancer, then induced gene expression should show decreased prostate cancer cell proliferation. It is also possible that inappropriate gene expression will induce apoptosis by markedly interfering with cell growth, particularly after several days. We will therefore directly determine apoptosis (TUNEL), proliferation (BrDU incorporation) as well as cell number and correlate these findings. For validated genes if antibody reagents useful for immunohistochemistry exist, we will consider carrying out immunostaining to assess gene expression in prostate tissue samples and correlate with prostate cancer clinicopathological data (months 30-36).

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April 6-10, 2013, Walter E. Washington Convention Center, Washington, DC

Abstract Number:	1974
Presentation Title:	Functional characterization of Basonuclin 1 (BNC1): a novel tumor suppressor gene commonly downregulated in human prostate cancer
Presentation Time:	Monday, Apr 08, 2013, 1:00 PM - 5:00 PM

Author Block: **Bernard Kwabi-Addo**[1], Songping Wang[1], Paulett Furbert-Harris[1], **Srinivasan Yegnasubramanian**[2], Joseph Devaney[3]. [1]Howard Univ. Cancer Ctr., Washington, DC; [2]Johns Hopkins Medical Institute, Baltimore, MD; [3]Children's National Medical Center, Washington, DC

Abstract Body: Prostate cancer (PCa) harbors a myriad of aberrant genomic and epigenetic alterations. Epigenetic inactivation of genes in PCa is largely based on transcriptional silencing by aberrant CpG methylation of CpG rich promoter regions. To better understand the role of aberrant methylation changes and to identify biological pathways likely to be affected in PCa, we used a genome-wide methylation array from Illumina (Infunium) to identify novel methylated genes associated to PCa. We identified several candidate genes including *BNC*1 as novel genes which are frequently methylated and whose methylation closely related to inactivation of gene expression in prostate cancer cell lines. Basonuclin1 is a zinc finger transcription factor that interacts with a subset of promoters of genes transcribed by both RNA polymerase-I and -II and has roles in maintaining ribosomal biogenesis and the proliferative potential of immature epithelial cells. We found that the expression of BNC1 negatively correlated with the degree of methylation at the CpG island in prostate tumor samples compared to benign prostate tissues. The forced-expression of BNC1 significantly decreased prostate cancer cell proliferation whereas the siRNA mediated BNC1 knock-down increased prostate cancer cell proliferation. Overall, our data suggest a potential tumor suppressor role for BNC1 that includes regulation of cell proliferation and cell-cell interaction in prostate carcinogenesis.

AACR American Association for Cancer Research | ANNUAL MEETING 2013
April 6-10, 2013, Walter E. Washington Convention Center, Washington, DC

Presentation Abstract #1985

Add to Itinerary

Presentation Title: ZNF783, a novel zinc finger protein has tumor suppressor function in prostate cancer

Author Block: Rana Tbaishat, Songping Wang, **Bernard Kwabi-Addo**. Howard Univ. Cancer Ctr., Washington, DC

Abstract Body: In the last decade hypermethylation of promoter regions has been accepted to be a prominent feature of human tumorigenesis by silencing tumor suppressor genes and other genes involved in cellular pathways. To study more in depth the involvement of aberrant methylation in prostate cancer tumorigenesis, we used a genome wide methylation array from Illumina (Infunium) to identify novel hypermethylated genes that might contribute to prostate cancer progression. *ZNF783* is one such candidate gene. *ZNF783* is Zinc-finger transcription factor that maps to human chromosome 7q33, a region implicated in prostate cancer tumorigenesis. We have observed significant hypermethylation of ZNF783 promoter CpG island in prostate cancer cell lines and prostate tumors when compared to match normal prostate tissue samples from radical prostatectomy patients. The treatment of prostate cancer cell lines with pharmacological demethylating agents restored ZNF783 gene expression indicating a role for epigenetic DNA methylation in regulating *ZNF783* expression in prostate cancer. The ZNF family of proteins has been identified to play essential roles in gene expression/regulation including inhibition of cell cycle proliferation, differentiation and apoptosis. The current study demonstrates a potential tumor suppressor role for ZNF783 in prostate cancer cells.

Prostate Cancer and Prostatic Disease (2013), 1–9

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ORIGINAL ARTICLE

Identification of novel DNA-methylated genes that correlate with human prostate cancer and high-grade prostatic intraepithelial neoplasia

JM Devaney[1], S Wang[2,3], S Funda[1], J Long[2], DJ Taghipour[2], R Tbaishat[3], P Furbert-Harris[2,4], M Ittmann[5] and B Kwabi-Addo[2,3]

BACKGROUND: Prostate cancer (PCa) harbors a myriad of genomic and epigenetic defects. Cytosine methylation of CpG-rich promoter DNA is an important mechanism of epigenetic gene inactivation in PCa. There is considerable amount of data to suggest that DNA methylation-based biomarkers may be useful for the early detection and diagnosis of PCa. In addition, candidate gene-based studies have shown an association between specific gene methylation and alterations and clinicopathologic indicators of poor prognosis in PCa.

METHODS: To more comprehensively identify DNA methylation alterations in PCa initiation and progression, we examined the methylation status of 485 577 CpG sites from regions with a broad spectrum of CpG densities, interrogating both gene-associated and non-associated regions using the recently developed Illumina 450K methylation platform.

RESULTS: In all, we selected 33 promoter-associated novel CpG sites that were differentially methylated in high-grade prostatic intraepithelial neoplasia and PCa in comparison with benign prostate tissue samples (false discovery rate-adjusted P-value <0.05; β-value $\geqslant 0.2$; fold change >1.5). Of the 33 genes, hierarchical clustering analysis demonstrated *BNC*1, *FZD*1, *RPL*39L, *SYN*2, *LMX*1B, *CXXC*5, *ZNF*783 and *CYB*5R2 as top candidate novel genes that are frequently methylated and whose methylation was associated with inactivation of gene expression in PCa cell lines. Pathway analysis of the genes with altered methylation patterns identified the involvement of a cancer-related network of genes whose activity may be regulated by *TP*53, *MYC*, *TNF*, *IL*1 and 6, *IFN*-γ and FOS in prostate pathogenesis.

CONCLUSION: Our genome-wide methylation profile shows epigenetic dysregulation of important regulatory signals in prostate carcinogenesis.

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Keywords: genome-wide DNA methylation analysis; pyrosequencing

INTRODUCTION

The progressive acquisition of both genetic and epigenetic defects is a major hallmark of human cancer, including prostate cancer (PCa).[1,2] For human PCa, abundant evidence has accumulated to suggest that somatic epigenetic alterations may appear earlier during cancer development, as well as more commonly and consistently, than genetic changes.[3] Furthermore, epigenetic changes tend to arise in association with age[4] and/or in response to chronic or recurrent inflammation, leading to cell and tissue damage.[5] Among the aberrant epigenetic defects are changes in cytosine methylation patterns and in chromatin structure and organization, which are equivalent to genetic changes in effecting and maintaining neoplastic and malignant phenotypes.[6] There is considerable amount of data to suggest that DNA hypermethylation may be useful for the early detection and diagnosis of PCa.[7] The CpG residues, which serves as targets of DNA methylation, have an asymmetric distribution in mammalian genomes and are often found in small clusters called CpG islands (CGIs).[8] Approximately 60% of all human gene promoters overlap with CGIs,[9] and accumulation of promoter DNA methylation is associated with gene silencing.[10] However, to date, only 115 genes are reported as methylation targets in PCa, 85 of which are listed in the Pubmeth database (http://www.pubmeth.org).[11] Methodologies that have been used to analyze DNA methylation changes in PCa have varied from a few CpG sites[12] to assessing several thousand genomic regions with a the CGI array.[13] The sodium bisulfite modification approach for distinguishing between methylated and unmethylated DNA has been used in combination with methylation-sensitive (for example, R. *Hpa*II) and -specific (for example, R. *Mcr*BCI) restriction enzymes, and/or affinity reagents specific for methylated DNA, such as the anti-5meC antibody or recombinant methyl-binding polypeptides.[14,15] However, these techniques require a high level of specialization and are still expensive and time-consuming. The advent of DNA methylation microarrays with a user-friendly component is now available. Among them is the recently developed Infunium human methylation array (Illumina Infinium Human Methylation 450 BeadChip) that offers high-throughput method for quantitatively assessing methylation across the genome and has been validated for both biology and function

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in colorectal cancer.[16] The goal of the present study is to apply this technique to identify new sites of methylation that can be correlated with the presence of PCa or result in silencing of genes previously not identified but functionally important for the initiation of PCa.

MATERIALS AND METHODS

Prostate cell lines

The immortalized normal prostate epithelial cell line RWPE1 and the human PCa cell lines PC3, DU145 and LNCaP, and BPH cell line were obtained from the American Type Culture Collection (Manassas, VA, USA). The immortalized normal prostate epithelial cell line pNT1A was obtained from the European Collection of Cell Culture (Salisbury, UK). The above cell lines were all derived from European American (EA) patient samples. The androgen-dependent PCa cell lines, E00D6AA, and androgen-independent cell line, MDA-PCa2b, were derived from African American (AA) PCa patients and obtained from ATCC. All cell lines are maintained in RPMI supplemented with fetal bovine serum (10%) (Invitrogen, Carlsbad, CA, USA) unless stated otherwise. The androgen-dependent PCa cell lines HPC1L and HPC8L were derived from anAA PCa patient at the Howard University under the Howard University Institutional Review Board approval. The HPC1L and HPC8L cell lines are positive for androgen receptor, PSA and epithelial membrane antigen. The HPC1L and HPC8L cells were maintained in RPMI supplemented with cholera toxin (2.5 ng ml^{-1}), epidermal growth factor (10 ng ml^{-1}), insulin (5 ng ml^{-1}), hydrocortisone (100 pg ml^{-1}), phosphoethanolamine (0.005 mM) and selenious acid (45 nM).

Human prostate tissue samples

Normal (benign prostate tissues, NL), high-grade prostatic intraepithelial neoplasia (HGPIN) and PCa (Gleason score 6–8) tissues, as well as pairs of matched NL and PCa tissues (same patient samples) were obtained with informed consent from the Baylor College SPORE tissue bank. The pathological status was confirmed by hematoxylin and eosin staining, and the cancer samples had a tumor cell percentage of 70–100% with Gleason scores of 6–8. All individual samples were obtained from EA patients (age 45–67 years old) who were either organ donors or had undergone radical prostatectomy.

Genome-wide methylation analysis

High molecular weight genomic DNA (0.5 μg) was bisulfite converted using the EZ DNA Methylation Gold kit (Zymo Research, Irvine, CA, USA). DNA was then whole-genome-amplified, enzymatically fragmented, precipitated, resuspended and hybridized at 48 °C for 16 h to an array containing 485 577 locus-specific oligonucleotide primers (Illumina, CA, USA). The probes were recognized at 20 216 transcripts, potential transcripts or isolated CGIs. After hybridization, the cytosine or thymine nucleotides were detected by fluorescent single-base primer extension using an Illumina Hi Scan SQ scanner. Initial array results were visualized using Illumina Genome Studio (Version 2011). The methylation score for each CpG site is represented as a β-value. The β-value is a continuous variable ranging between 0 and 1, representing the ratio of the intensity of the methylated-probe signal to the total locus signal intensity. A β-value of 0 corresponds to no methylation, whereas a value of 1 corresponds to 100% methylation at the specific CpG site measured. *P*-values were calculated to identify failed probes as per Illumina's recommendations and no arrays exceeded our quality threshold of $>5\%$ failed probes. In addition, we removed CpG sites on the X and Y chromosome[17–19] and removed CpG sites from the analysis that contained a single-nucleotide polymorphism or a single-nucleotide polymorphism within 10 base pairs of the methylation probe[17] according to dpSNP132. We only focused on CpG sites within known CGIs from the UCSC (University of California, San Francisco) database ($n = 200\,419$ CpG sites). The sites in the UCSC database were discovered using a modified method from Gardiner-Garden and Frommer.[20] Raw data were normalized using Illumina's control probe scaling procedure[17] and background subtracted.

The β-values were imported into Partek Genomics Suite (version 6.6; Partek, St Louis, MO, USA) and underwent a logit transformation (*M*-value).[21] The *M*-value is calculated as the log2 ratio of the intensities of the methylation probes versus unmethylated probe. The problem of heteroscedasticity in the high and low ranges of methylation (<0.2 and $\geqslant 0.8$) is resolved with the transformation of the β-value to *M*-value. In addition, we removed noise from our analysis by examining only CGIs with a β-value of $\geqslant 0.1$. The data were analyzed using a Student's *t*-test between NL, HGPIN and PCa. The *M*-value score was treated as a continuous variable that is the response for the analysis of variance model. An false discovery rate correction was implemented, but none of the identified CGIs were significant. Therefore, we validated the results with pyrosequencing.

These β-values were used to calculate a ratio of relative methylation between samples, with higher values corresponding to greater levels of methylation (hypermethylation) in tumor tissue relative to normal. Hypomethylation is defined as methylation in normal prostate samples but undermethylated in tumor samples. The CpG methylation differences were considered significant above a cut-off *P*–value $<10^{-3}$ and 0.2-fold change in the β-value, unless specifically indicated otherwise. Unsupervised hierarchical clustering analysis of the most variable β-values was done with a false discovery rate-adjusted *P*-value <0.05as significant. All other computations and statistical analyses were performed using Partek Genome Studio.

Pyrosequencing

High-molecular-weight genomic DNA was bisulfite converted using Epitect Bisulfite kit (Qiagen, Valencia, CA, USA). Pyrosequencing was done using the PSQ HS96 Gold SNP Reagents on a PSQ 96HS machine. Primers were designed in-house or commercially available PyroMark CpG assays consisting of PCR and sequencing primers were used according to the manufacturer's protocol (Qiagen). Epitect ready-to-use, completely methylated and bisulfite-converted human control DNA was used as a positive control and unmethylated and bisulfite-converted human control DNA was used as a negative control. Each bisulfite PCR and pyrosequencing reaction was done at least twice.

Gene expression

Total RNA extracted from cells and prostate tissues using TRIzoL Reagent (Invitrogen) was used in cDNA synthesis using Invitrogen SuperScript first strand synthesis system for reverse transcriptase-PCR and according to the manufacturer's protocol (primers used are listed in Table 3). Either SYBR green or TaqMan assay (designed for *BNC*1, *FZD*1, *CYB*5R2 and *SYN*2) were used to quantitatively measure mRNA expression. Real-time PCR was carried out in a Bio-Rad iCycler real-time thermal cycler (Biorad, Hercules, CA, USA) as described previously,[22] and incorporating optimized PCR reaction conditions for each gene. The threshold cycle (C_t) in the PCR cycle at which fluorescence exceeds background was then converted to copy number based on a cDNA standard curve generated. Each experiment was carried out in duplicate.

Treatment with 5-aza-2′-deoxycytidine and/or trichostatin A

The pNT1A, DU145, PC3 and LNCaP cells were seeded at 5×10^5 cells per 100-mm tissue culture dish. After 24 h of incubation, the culture media was changed to a media containing 5′-aza-dC (5-aza-2′-deoxycytidine; 5 μM) for 72 h and/or trichostatin A (TSA; 250 nM) for an additional 24 h. Total RNA extracted from cells using TRIzol Reagent (Invitrogen) was first used in first strand DNA (cDNA) synthesis using Invitrogen Super-Script first-strand synthesis and then used in real-time quantitative PCR as previously described.[23] Mock-treated cells were cultured similarly. Three independent experiments were carried out for each analysis.

Pathway analysis

Biological functional categorization and pathway construction were performed using the Ingenuity Pathway Analysis software tool produced by Ingenuity systems. Ingenuity Pathway Analysis utilizes an extensive database of functional interactions, which are drawn from peer-reviewed publications and manually maintained.[24]

Statistical Analyses

The methylation index at each gene promoter and for each sample was calculated as the average value of mC/ (mC + C) for all examined CpG sites in the gene and was expressed as the percentage of methylation. Data analysis was done using either Prism 4 software (GraphPad Software) or SPSS for Windows (version 13.0, SPSS). Comparison of grouped samples β was done using analysis of variance for NL, PCa and HGPIN, and age as categorical variables with fixed effects. For each gene, the frequencies of methylated and unmethylated cases were analyzed using rank-sum

Mann–Whitney's *U*-test to compare each group in pair-wise fashion. Resulting *P*-values were adjusted for multiple comparisons using the Bonferroni-adjusted Mann–Whitney's *U*-test. The non-adjusted statisitcal level of significance of $P<0.05$ corresponds to a Bonferroni-adjusted statistical significance of $P<0.0167$.

RESULTS

450K DNA methylation analysis of genomic DNA from prostate tissues

In order to find CpG sites that are differentially methylated in NL, high-grade prostatic intraepithelial neoplasia (HGPIN) and PCa tissue samples, we used the Illumina Infinium platform to conduct a genome-wide large-scale analysis of DNA methylation changes. For our analysis, we included six NL, seven HGPIN and six PCa tissue samples from EA men who are either organ donors or had undergone radical prostatectomy. Unsupervised hierarchical clustering analysis of all samples (Figure 1) shows heterogeneity in individual prostate tissue samples. However, most normal prostate tissue samples (five out of six cases) clustered together, whereas most HGPIN and PCa cases generally clustered together. We separated the methylation arrary data into three subsets. The first subset consisted of six PCa samples compared with six NL samples, the second subset consisted of seven HGPIN samples compared with six NL samples and the third subset consisted of six PCa samples compared with seven HGPIN samples. Table 1 shows the results of the most significant differentially methylated genes identified in our analysis. We observed 1317 CpG sites to be hypermethylated and 44 CpG sites to be hypomethylated in PCa tissues when compared with normal prostate tissues. The HGPIN samples showed 167 CpG sites to be hypermethylated and 312 CpGs to be hypomethylated when compared with the normal prostate tissues. In addition, we observed 138 hypermethylated CpG sites and 7 hypomethylated CpG sites when we compared PCa tissues with HGPIN. As expected, CpG sites corresponding to genes that were previously shown to be hypermethylated in PCa were also hypermethylated in our samples, including *GSTP*I, *RASSF*1A and *NKX*2-5.[4,25] Our analysis shows high frequency of hypermethylation in PCa tissues in comparison with normal prostate tissues. Our observation of more hypomethylation than hypermethylation sites from the array data in HGPIN versus normal prostate is consistent with other published studies that reported genome-wide DNA hypomethylation as prevalent as hypermethylation,[26,27] with substantial DNA hypomethylation in genes of cancer cells compared with their normal counterparts.[28] However, differences in the hypermethylation and hypomethylation frequencies observed in PCa versus normal prostate tissues when compared with HGPIN versus normal prostate tissues may reflect the random variability in tissue composition or random nature of affected methylation sequence.

Overall, several of these CpG sites are located in the genomic regions that are not associated with gene promoter loci (data not shown). Previous reports indicate that the distribution of the CpG content in the human genome is estimated to be 30.9% in CGIs, 23% in CpG shores, 9.7% in CpG shelves and 36.3% are isolated CpGs in the genome defined as 'open sea'.[16] We excluded genes without CGIs in the promoter and imprinted genes,[29] and focused on CpG-methylated sites in proximal promoters defined as the sum of CpG sites located within 200 bp downstream or 1500 bp upstream of the transcription start site in order to assess the effects of promoter methylation on gene expression. From a functional genome distribution

Table 1. Number of methylated CpG sites identified in comparative analysis of the Illumina Infinium genome-wide DNA methylation array

	PCa versus NL	*HGPIN versus NL*	*PCa versus HGPIN*
Hypermethylation	1317	167	138
Hypomethylation	44	312	7

Abbreviations: HGPIN, high-grade prostatic intraepithelial neoplasia; NL, normal; PCa, prostate cancer.
European American patients samples used are as follows: PCa (six PCa tissues) versus NL (six NL/benign prostate tissues); HGPIN (seven) versus NL; or HGPIN versus PCa. *P*-values <0.005 and β-value of 0.2 and above are statistically significant.

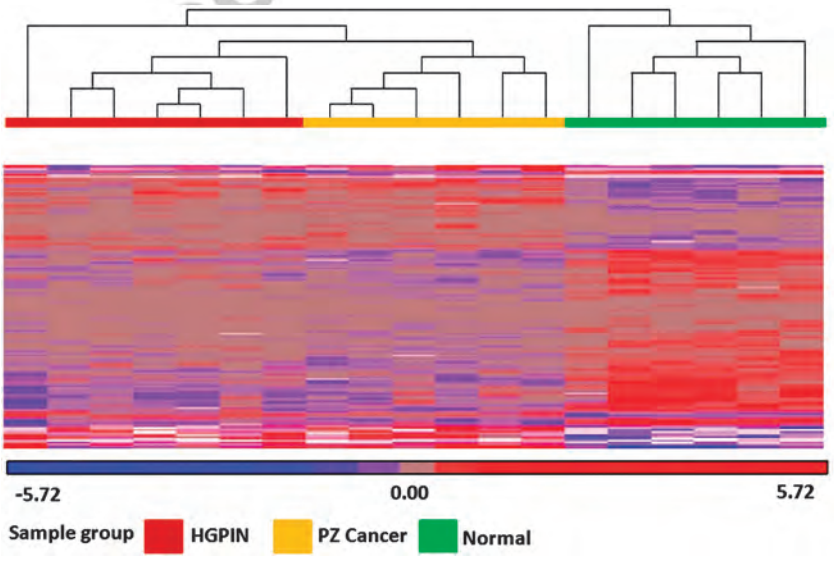

Figure 1. Hierarchical cluster analysis of methylation findings from the microarray data. DNA methylation hierarchical clustering shows high within-sample group similarity and between-samples heterogeneity. These analyses were done using Partek genome studio software with 450 gene probes showing greatest variability across all samples. CpG methylation differences were considered significant above a cut-off *P*-value $<10^{-3}$ and 0.2-fold change in the β-value. The scale is based on β-value (*M*-value; as determined by Partek Genome Studio) for methylation score.

Table 2. The gene name, chromosomal location and function of the top 33 novel genes whose proximal promoters show differential hypermethylation in association with the progression of PCa from benign state to HGPIN or PCa as determined by the Illumina Infinium genome-wide DNA methylation array

Gene abbreviation	*Gene name*	*Locus*	*Function*
ALDH1L1	Aldehyde dehydrogenase 1 family member L1	3q21.3	*De novo* purine biosynthesis etc
ALDH5A1	Aldehyde dehydrogenase 5 family member A1	6p22.3	Degradation of the inhibitory neurotransmitter GABA
ADARB2	RAN-specific adenosine deaminase	10p15.3	Role in RNA editing
BNC1	Basonuclin 1	15q25.2	Transcription factor
CELF2	CUG triplet repeat RNA-binding protein	10p13	Post-transcriptional regulation
CHRNB1	Cholinergic receptor, nicotinic beta 1	17p13.1	Nicotinic receptor
CYB5R2	Cytochrome b5 reductase	11p15.4	Drug metabolism, cholesterol biosynthesis
CXXC5	CXXC-type zinc finger protein	5q31.2	NF-κB/MAPK pathway
EPDR1	Ependymin related protein 1	7p14.1	Neuronal regeneration
EPHA5	Ephrin type A receptor 5	4q13.1	Receptor tyrosine kinase
FZD1	Frizzled family receptor 1	7q21.13	Wnt signaling
GNG7	Guanine nucleotide binding protein γ7	19p13.3	Transducer in various transmembrane signaling system
HES2	Hairy and enhancer of split 2	1p36.31	Transcription repression
ITPKA	Trisphosphate 3-kinase A	15q15.1	Regulates inositol phosphate metabolism
JAK2	Janus kinase 2	9p24.1	Cytokine receptor signaling
LEO1	Component of Paf1/RNA polymerase II comples	15q21,2	RNA processing
LMX1B	LIM homeobox transcription factor 1, beta	9q33.3	Transcription Factor
MAFB	Musculoaponeurotic fibrosarcoma oncogene homolog B	20q11.1-q13.1	Transcriptional activator/repressor
MYO5C	Myosin VC	15q21.2	Transferrin Trafficking
NOS1	NO synthase 1	12q24.22	Produces NO
OTP	Orthopedia homeobox	5q14.1	Differentiation of hypothalamic neuroendocrine cells
PAQR5	Progestin and adipoQ receptor family member v	15q22.31	Progesterone signaling
PCSK9	Proprotein convertase subtilism type 9	1p32.3	Cholesterol homeostasis
PLXNC1	Plexin C1	12q23.2	Viral receptor
PURG	Purine rich element binding protein G	8p11	Transcription initiation
RPL39l	Ribosomal protein L39-like protein	3q27.3	Related to ribosome
SERPINB9	Serpin peptidase inhibitor, clade B member 9	6p25.2	Granzyme B inhibitor
SHC4	Src homology 2 domain containing family member 4	15q21.1-q21.2	Ras signaling
SLC25L24	Solute carrier family 25 member 24	1p13.3	Calcium-dependent mitochondrial solute carrier
SYN2	Synapsin 2	3p25.2	Neurotransmitter regulation
TRIM15	Tripartite	6p21.3	Unknown
ZNF783	Zinc finger family member 783	7q36.1	Transcriptional regulation
ZFP91	Zinc finger protein 91	11q12.1	Ubiquitination

Abbreviations: GABA, γ-aminobutyric acid; HGPIN, high-grade prostatic intraepithelial neoplasia; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor-κB; NO, nitric oxide; PCa, prostate cancer.

standpoint, these proximal promoters account for 41% of the CpGs in the human genome.[16] Using this approach, we selected the top 33 novel genes that are common and differentially methylated in PCa versus NL and HGPIN versus NL prostate tissues (Table 2).

Pyrosequencing analysis of novel methylated genes

To confirm the accuracy of the Illumina methylation array, we used pyrosequencing to analyze the methylation status of the 33 novel genes in normal and PCa cell lines derived from both EA and AA men. For pyrosequencing, we used both commercially designed primers from Qiagen and primers that were designed in-house (see Table 3 for primers detail). A heat map hierarchical cluster analysis for the methylation status in the prostate cell lines is shown in Figure 2. The results demonstrated that the 33 novel genes were methylated in at least 1 PCa cell line with higher methylation frequency for the majority of the genes analyzed in the PCa cell lines when compared with the primary immortalized normal prostate cell lines. Interestingly, we observed higher methylation frequency in the PCa cell lines that were derived from the AA when compared with EA PCa cell lines. This observation is consistent with a previously published study,[30] where we reported significantly higher methylation frequency in human prostate tissues samples from AA patients in comparison with EA patients, clearly demonstrating that differential methylation patterns in AA and EA men may result in altered patterns of gene expression and contribute to PCa disparity. Overall, we identified *BNC*1, *FZD*1, *RPL*39L, *SYN*2, *LMX*1B, *CXXC*5, *ZNF*783 and *CYB*5R2 as top candidate genes that are frequently hypermethylated in PCa cell lines.

Validation of methylated genes in PCa progression

To determine whether the genes that were hypermethylated in PCa cell lines were hypermethylated in a cancer-specific manner, we analyzed the methylation status of the 8 top novel genes in the samples used in the array analysis, and additional 23 NL and 17 PCa that were available. The methylation frequency and mean are shown in Figure 3a. In general, statistically significant differences ($P<0.05$) in the methylation frequencies were noted in PCa samples in comparison with the benign prostate tissues for *BNC*1, *FZD*1, *SYN*2, *ZNF*783 and *CYB*5R2 genes. Significant differences were also noted for HGPIN samples in comparison with benign prostate tissues for *SYN*2 and *CYB*5R2 genes. However, no significant differences in the methylation frequency were noted

Table 3. (A) List of primer sequences used for pyrosequencing analysis of methylation status; (B) PyroMark CpG Assays consisting of PCR and sequencing primers

(A) Pyrosequencing

	Sense (5′–3′)	Antisense (5′–3′)	Sequencing primer (5′–3′)
ADARB2	AGTTATATGGTGTATATATTTTGGATTAAG TTGGGAATTGGTATGGA	U-AAAACCTCTTCCTAAAAACAAACC and 5′-Biotin-U	
ALDH1L1	AGAGGGAGGTAGGATAGTAGATTTTATA GGTTTAGTTATAGAGAGTTTAGGTT	U-AATTCTTTCCTCTCCTAAACAAATAC and 5′-Biotin-U	
ALDH5A1	GGGAGTAATTTTAGGAGAATGTTTAGATA AAAAAGTAGTTAGGTAGTAGA	U-ACCTACCCTAAATACCAAAACC and 5′-Biotin-U	
BNC1	TGAAGGTATTTGTTGGTAAAAGATAT	Biotin-AAAAAAAAAAACCCAATCATCTCCTAAA	GGTATTTGTTGGTAAAAGATATAG
CXXC5	TGTGTGTGTATGGTGTATGT AAGTGTGTGTTTGGG	U-CTCAAAAAACCTAAATCACCCCATCCC and 5′-Biotin-U	
CYB5R2	GGTTTTGGGTTAGTTTTGTTTTTAGG	Biotin-ACCTTCTCCAACCTTACC	GGTTAGTTTTGTTTTTAGGG
FZD1	GGGAAGTTTTAGTATTGTTTTTAAAGATG ATTTTATTTTGTTTTTTGGAATTA	U-CCTCTCCTCCCAAAATCTCT and 5′Biotin-U	
GNG7	ATGAGGTTTGTTTTTTTTTTAAAAGTTAAT AAAGTTAATAGTTGGTGAAT	U-CCCTAAACTCCTACTTTTTTCC and 5′Biotin-U	
HES2	GGATAGTAGAGGGAGGGATTTGTATTTTTA GGATTTGTATTTTTAGTTGTTTTAT	U-ACTAAAACCCTTATCCAAACT and 5′Biotin-U	
ITPKA	AATGTTGGAGGTGGATTTTGAAGTT	Biotin-CCTACCTCACCTTAATACCC	AGTTTTTAAGGAGGAGGAGT
LMX1B	GGGATTGATTAGAAGAGAGAGGT GTTGGATTTTTGGGATTAA	U-AAATAACCAACCCTAAAAACCTAC and 5′-Biotin-U	
MAFB	GGTTTAGGGTTATAGGGTGAGAAATTTT GGTTATAGGGTGAGAAATTTTT	U-CCCAAACCCAATCTAAATACCT and 5′-Biotin-U	
OTP	TGGGGTTAGTATAGATTTTAGGGTATAG ATTTTAGGAGTAGGTTTATTGAGT	U-CATATTAACCCCCCAACCCAAAAC and 5′-Biotin-U	
RPL39L	AGTAGGTTTGAGTTGTGGTATTG TGTGGTATTGTTGTTTTTAG	U-ATAAATAAAAATTCCTCCTCACC and 5′-Biotin-U	
SERPINB9	AAGAGGTGTTTGTAGAGGTTTTAG GGGGTAGGAGTTTAGGTT	U-CCCTCACCTACCACCTAAT and 5′-Biotin-U	
SYN2	AGGTTTAGTTGGGAGGAGGT GGAGGTGAGGGTTGAG	U-ACTATTTCCTAAAAACCTTTCCTATTAC and 5′-Biotin-U	
TRIM15	AGGTTTTTGTTTTAAGTTGAAAGGTTGAAG AAAGGTTGAAGTGGG	U-AACTCAAATCCCCTATTCTCT and 5′-Biotin-U	
ZNF783	ATGGGAAGGGATAGGTTTAG TTTAGGGTTAAGTATTTATTAGAGG	U-CTCTCTTATAAACCACACAATCTTTCTCT and 5′-Biotin-U	

(B) PyroMark CpG assays (commercially available primers from Qiagen)

PM00092981	Hs_PCSK9_01_PM
PM00029078	Hs_EPDR1_01_PM
PM00145775	Hs_CELF2_01_PM
PM00159383	Hs_PLXNC1_01_PM
PM00036911	Hs_PURG_01_PM
PM00068327	Hs_CHRNB1_01_PM
PM00018683	Hs_EPHA5_01_PM
PM00050974	Hs_NOS1_01_PM
PM00058436	Hs_MYO5C_01_PM
PM00139916	Hs_JAK2_01_PM
PM00169743	Hs_LEO1_01_PM
PM00168525	Hs_PAQR5_01_PM
PM00049861	Hs_ZFP91_01_PM
PM00170947	Hs_SHC4_01_PM
PM00106351	Hs_SLC25A26_01_PM

Abbreviation: U, universal primer sequence.
Primer sequences above used in the pyrosequencing analysis were designed in-house. U represents universal primer sequence- *5′*-GGGACACCGCT GATCGTTTA-*3′*.

for the HGPIN tissue samples in comparison with the PCa tissues. Our data clearly demonstrate that the methylation levels of these novel genes can distinguish between benign and HGPIN or PCa tissues. However, we need a larger sample size to confirm these preliminary data.

To investigate whether methylation is associated with gene expression in PCa tissues, we performed expression analysis by quantitative reverse trascriptase-PCR for BNC1 and CYB5R2. The results presented in Figure 3b showed a significantly higher level of gene expression for BNC1 ($P<0.0001$) and CYB5R2 ($P=0.0004$) in the matched NL when compared with the PCa tissues. Our data suggest an inverse association between DNA methylation and gene expression for BNC1 and CYBR52 (cytochrome b(5) reductase 2). We observed that in cancer samples that showed higher methylation, this was associated with low levels of gene expression, whereas the normal prostate

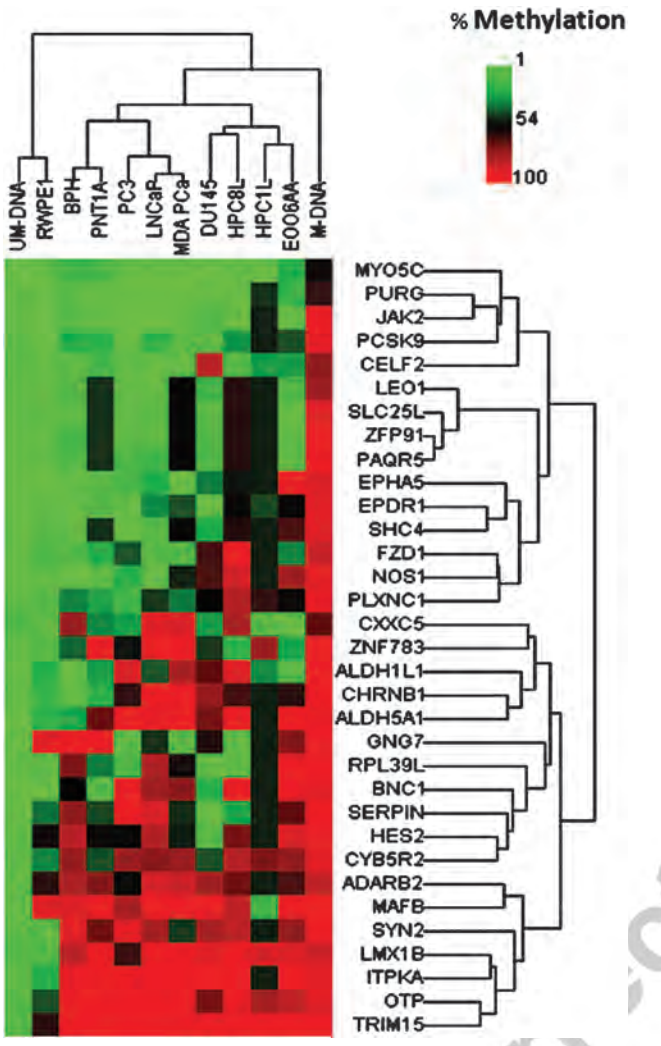

Figure 2. Hierarchical cluster analysis of gene validation of 33 novel selected promoter-associated DNA-methylated genes in a panel of prostate cell lines: RWPE1, primary immortalized prostatic epithelial cells; PNT1A, primary immortalized epithelial cells; BPH; European American (EA) prostate cancer (PCa) cell lines are LNCaP, PC3 and DU145; African American (AA) PCa cell lines are MDA PCa2b, HPC1L, HPC8L and E006AA. Percent methylated cytosines in the cell lines are obtained by pyrosequencing. Methylated DNA control (M-DNA) and unmethylated DNA control (UM-DNA) were purchased from Qiagen.

samples had lower methylation and higher gene expression level to indicate that methylation leads to some loss of gene expression.

Gene expression and induction after 5-aza-dC treatment in prostate cell lines

To confirm that hypermethylation affects gene expression, we tested the hypothesis that pharmacological modulation of methylation can reactive gene expression,[31] PCa cell lines were treated with the demethylating agent 5′-Aza-dC with or without the histone deacetylase inhibitor, TSA. Gene expression was analyzed by quantitative reverse trascriptase-PCR using untreated and treated cells to assess whether promoter CGI hypermethylation was closely associated with gene expression in the newly identified cancer-specific methylated genes (Figure 4). Treatment with 5′-Aza-dC, an inhibitor of the DNA methyltransferase,[31] caused over twofold increase in mRNA transcript expression levels for BNC1, CYB5R2 and FZD*1*, and a modest increase in the mRNA transcript level for SYN2. Previous reports have suggested that histone deacetylase cooperates with methyl-binding proteins at hypermethylation CGIs to repress transcription,[32,33] and Cameron *et al.*[34] have reported that methylation-induced transcriptional repression could be overcome with a combination of 5′Aza-dC and TSA. We have found that treatment with TSA also modestly increased the mRNA transcript levels for BNC1 and FZD1; however, the combined 5′Aza-dC and TSA treatment synergistically increased the mRNA transcripts for all genes tested in both the androgen-dependent (LNCaP) and the androgen-independent (PC3) cell lines. The results indicate that epigenetic mechanisms, including DNA methylation, are likely involved in the reduced expression of BNC1, CYB5R2, FZD1 and SYN2.

DISCUSSION

With advancements in microarray technology, the number of genes found to be hypermethylated in PCa in a cancer-specific manner is expected to increase exponentially, leading to a better understanding of epigenetic modulation of tumor-related genes in prostate carcinogenesis. To identify novel methylated genes associated with PCa progression, we have applied the Illumina Infinium human genome-wide DNA methylation array that consists of 485 577 CpG sites using prostate tissue samples corresponding to benign, HGPIN and PCa tissues.

We present the identification of several novel genes demonstrating cancer-specific methylation in PCa through the use of Infinium arrays. The fidelity of the Illumina Infinium array was confirmed using pyrosequencing for genes selected according to our criteria described above. As a result, 33 novel promoter-associated CpG sites were observed to be differentially methylated in HGPIN and PCa in the DNA array analysis. Most of the genes identified through our arrary analysis have either never been shown to be methylated in PCa or in other cancer types. Previously reported methylated genes in PCa, such as *GSTP*I, *RASSF*1A and *NKX*2-5, all showed evidence of methylation based on fold changes and statistical significance. However, the stringency of the statistical analyses that we performed may have excluded these genes within our top genes of the cancer/reference data set. The methylation status of all 33 genes were validated by pyrosequencing in PCa cell lines, with at least two out of the eight PCa cell lines showing hypermethylation in comparison with the immortalized primary epithelial prostate cell lines (RWPE1) for these genes. Eight out of the 33 genes were selected for methylation validation in human prostate tissues, because they were frequently hypermethylated in the PCa cell lines. The promoter-associated CpG sites for *BNC*1, *FZD*1, *SYN*2, *ZNF*783 and *CYB*5R2 genes demonstrated significant differences in methylation level in HGPIN and PCa when compared with the NL prostate tissues; therefore, we tested whether pharmacological modulation can give reactive gene expression, which would be indicative of methylation having a role in gene expression (we did not investigate ZNF783, because it should hypermethylation even in the NL samples). Treatment of PCa cell lines with 5-aza-dc and TSA drugs induced gene re-expression; therefore, these genes are considered to be repressed by DNA hypermethylation in their promoter CGI, suggesting they can be potentially explored as novel markers for disease identification. These novel genes have diverse roles in normal and PCa pathogenesis, which underscore the myriad way these epigenetic aberrations may contribute to PCa disease etiology and/or progression. For instance, *BNC*1 belongs to the basonuclin gene family, which encodes for zinc finger proteins mainly expressed in the epidermis, hair follicles and the germ cells of the testis and ovary.[35] The BNC1 protein is an unusual transcription factor that interacts with a subset of

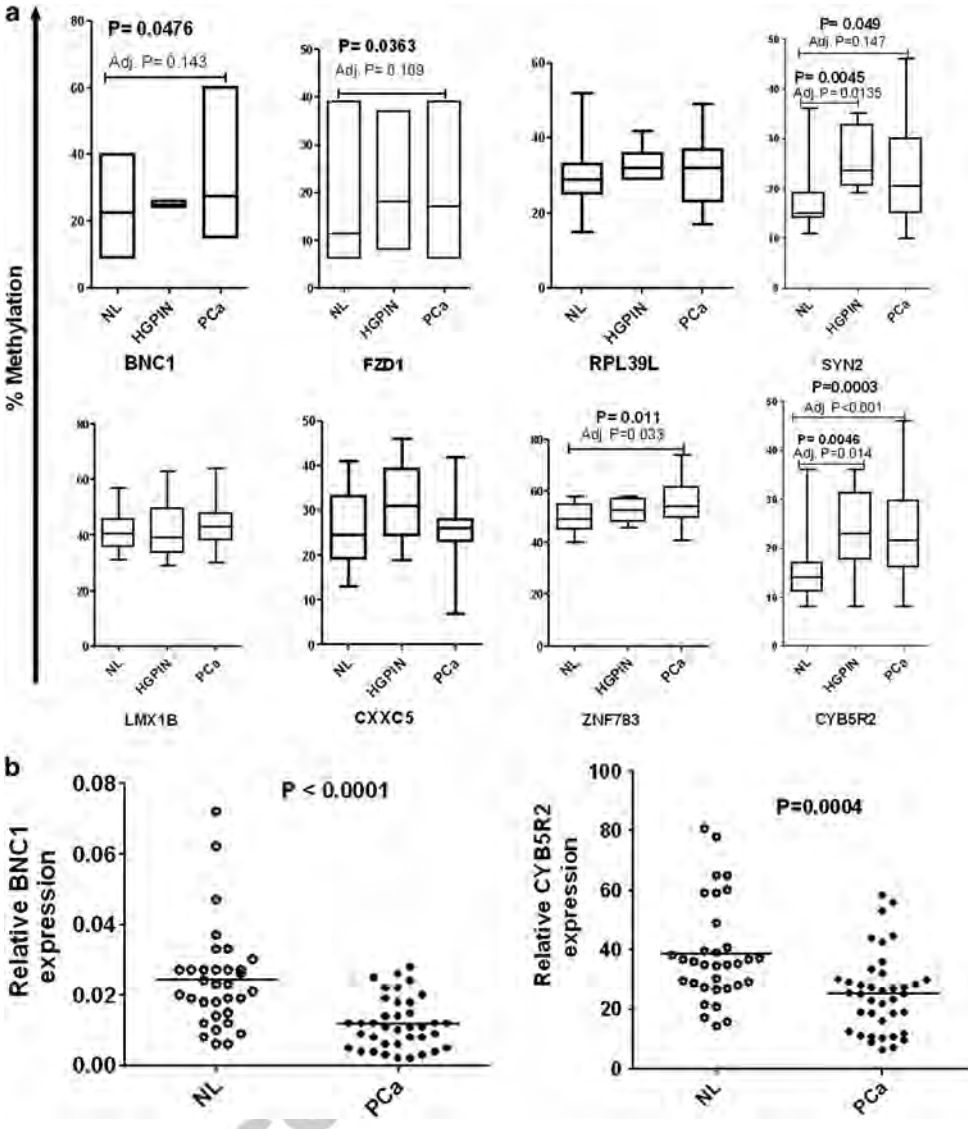

Figure 3. (**a**) Quantitative DNA methylation analysis in human prostate tissues is shown in the box plot. The percent DNA methylation levels of promoter CGIs were analyzed in bisulfite-modified genomic DNA extracted from normal (NL, 29 samples), high-grade prostatic intraepithelial neoplasia (HGPIN; 7 samples) and prostate cancer (CA, 23 samples) tissue samples. The *y* axis, percentage of methylated cytosines in the samples as obtained from pyrosequencing; *x* axis, normal, HGPIN and CA tissues. The box plot describes the median, interquartile range and maximum/minimum methylation. (**b**) Gene expression in prostate tissue samples. The relative mRNA transcript expression levels for BNC1 and CYBR52 were analyzed in 32 matched pairs of NL (normal) and PCa (prostate cancer) tissue samples by reverse trascriptase-PCR and expressed relative to β-actin to correct for variation in amount of reverse-transcribed RNA. *P*-value where significant ($P < 0.05$; Mann–Whitney's *t*-test) is shown. Adj. P, adjusted *P*-value (Bonferroni).

promoters of genes that are transcribed by both RNA polymerase-I and -II, and has roles in maintaining ribosomal biogenesis and the proliferative potential of immature epithelial cells.[36] Methylation of *BNC*1 has previously been reported in renal cell carcinoma[37] and in chronic lymphocytic leukemia.[38] Here we describe the methylation and silencing of *BNC*1 in PCa tissues. Our observation supports previous reports of frequent methylation of *BNC*1 across common epithelial cancers. Similarly, Wnt/Frizzleds (FZD; including FZD1) signaling is known to have a key role in development, tissue-specific stem cell maintenance and tumorigenesis, particularly through the canonical pathway involving stabilization of β-catenin.[39] Studies in mouse models have suggested a role for Wnt/FZD signaling in promoting tumorigenesis.[39] Here we describe the methylation of *FZD*1 in PCa. One group has previously reported the age-dependent methylation of *FZD*1 by whole human genome analysis.[40]. Other groups have reported aberrant expression of FZD1 in colon cancer,[41] breast cancer cells[42] and in neuroblastoma,[43] suggesting a potential role for FZD1 in processes associated with tumor invasion. However, some groups have reported increased expression of FZDs (including FZD1) in cancer tissues.[41–43] Although it is not clear why FZD1 expression may parallel DNA methylation changes, increased DNA methylation in the gene bodies of expressed genes has been reported and may have a role in the gene transcription[44,45] On the other hand, loss of the secreted Fzd-related protein 1 and 2 (SFRP1 and -2) expression by hypermethylation has been reported in human cancers, including PCa[46] and breast cancer tissues[47] to suggest that hypermethylation of SFRPs may be a common phenomenon in tumorigenesis. Another novel gene that we have identified is *CYB5*R2 gene, which belongs to the family of

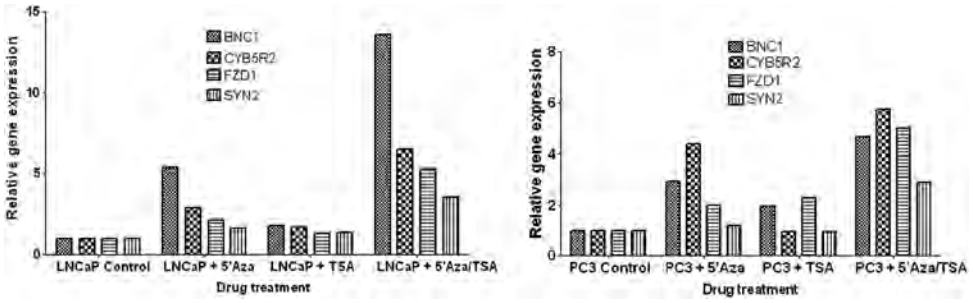

Figure 4. Demethylation and gene expression. The androgen-dependent prostate cancer (PCa) cell line, LNCaP, and the androgen-independent PCa cell line, PC3, were treated with 5′-aza-dC (5-aza-2′-deoxycytidine; 5 μmol l^{-1}), trachostatin A (TSA; 250 nmol l^{-1}), or a combination of the two drugs. This cell line had high methylation levels for the genes tested and all had very low levels of expression at baseline. The gene expression was determined by quantitative reverse trascriptase-PCR and expressed relative to β-actin to correct for variation in the amounts of reverse-transcribed RNA. The data are representative of three independent experiments. The fold change in gene expression level was calculated relative to the control (untreated cells) that was set at an arbitrary unit of 1.

drug-metabolizing enzymes that are responsible for the activation, inactivation and detoxification of many chemotherapeutic agents, and deficiencies in these enzymes may result in bioavailability and disposition of these agents.[48] Several studies have shown polymorphisms in genes encoding these drug-metabolizing enzymes and association with response to cancer treatment.[49] The most important polymorphisms shown to influence survival after cancer treatment are polymorphisms in the genes encoding the phase II detoxification enzymes glutathione-*S*-transferase (including glutathione *S*-transferase pi; GSTP1). In PCa, polymorphisms do not appear to be associated with increased PCa risk, but promoter methylation of GSTP1 has been reported as frequent alteration in the disease etiology and progression.[50] To our knowledge, this is the first report to indicate that CYB5R2 is also dysregulated in PCa by DNA methylation. The sensitivity of *BNC*1, *FZD*1, *SYN*2 and *CYB5R*2 methylation were similar to other well-known methylated genes, including *GSTP*1, *RARβ*2 and *RASSF*1A, which we have tested using the pyrosequencing technique.[4] Our analysis also investigated methylation differences in HGPIN in comparison with PCa tissues. Although HGPIN displays intermediate methylation frequencies between benign prostate tissues and PCa for several genes that is consistent with previous reports,[51,52] we did not observe any significant differences in the methylation frequencies for the HGPIN tissues in comparison with the PCa tissues. Previous reports have demonstrated significant differences in the methylation frequencies for several genes, including *RARβ2*, *RASSF*1A, *GSTP*1 in HGPIN in comparison with PCa.[53,54] Our result is probably due to the smaller sample size used in the current studies and perhaps a narrow spectrum of pathologic tumor stages that did not allow for a more powerful statistical analysis. In addition, one study that surveyed a panel of nine gene promoters only found four genes with methylation levels that differed significantly between HGPIN and PCa,[53] suggesting that hypermethylation of specific gene promoters maybe associated with advanced prostate tumor grade and stage.

In summary, we have identified 33 novel cancer-specific methylated markers for PCa using Illumina Infinium array and pyrosequencing. Of these *BNC*1, *FZD*1, *SYN*2, *ZNF*783 and *CYB*5R2 were frequently hypermethylated in a PCa tissue-specific manner, and their promoter hypermethylation was correlated with inactivation of gene expression in cell lines. Differential methylation has significant potential for clinical use as a biomarker, as methylation is stable in formalin-fixed parafin-embedded tissues and can be detected in very small numbers of cells using PCR-based techniques. Further studies will look into the implications of these newly identified DNA methylation markers for patient outcome in a large scale study of PCa samples.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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